

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER P66141US0
		US APPLICATION NO (if known, see 37 CFR 1.5) 09/701583
INTERNATIONAL APPLICATION NO PCT/EP99/04013	INTERNATIONAL FILING DATE 10 June 1999	PRIORITY DATE CLAIMED 10 June 1998
TITLE OF INVENTION A METHOD FOR STIMULATING THE IMMUNE SYSTEM		
APPLICANT(S) FOR DO/EO/US Karl-Hermann SCHLINGENSIEPEN, Reimar SCHLINGENSIEPEN and Wolfgang BRYSCH		

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - International Search Report - EPO
 - PCT/IB/301 Form
 - PCT/IB/304 Form
 - PCT/IB/308 Form
 - International Preliminary Examination Report - with no annexes
 - First Page of Publication

US APPLICATION NO (If known, see 37 CFR 1.5) <div style="font-size: 24pt; font-weight: bold; margin-top: 5px;">09/701583</div>		INTERNATIONAL APPLICATION NO <div style="font-weight: bold; margin-top: 5px;">PCT/EP99/04013</div>		ATTORNEY'S DOCKET NUMBER <div style="font-weight: bold; margin-top: 5px;">P66141US0</div>	
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17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) . . \$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . \$710.00 Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) \$1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) \$100.00 Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) \$860.00 <div style="text-align: right; font-weight: bold;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>	CALCULATIONS	PTO USE ONLY																																																				
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<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Claims</th> <th style="width: 20%;">Number Filed</th> <th style="width: 20%;">Number Extra</th> <th style="width: 20%;">Rate</th> </tr> <tr> <td>Total Claims</td> <td style="text-align: center;">13 - 20 =</td> <td style="text-align: center;">-0-</td> <td style="text-align: center;">x \$18.00</td> </tr> <tr> <td>Independent Claims</td> <td style="text-align: center;">2 - 3 =</td> <td style="text-align: center;">-0-</td> <td style="text-align: center;">x \$80.00</td> </tr> <tr> <td colspan="3">Multiple Dependent Claim(s) (if applicable)</td> <td style="text-align: center;">+ \$270.00</td> </tr> <tr> <td colspan="3" style="text-align: right; font-weight: bold;">TOTAL OF ABOVE CALCULATIONS =</td> <td style="text-align: center;">\$ 990.00</td> </tr> <tr> <td colspan="3" style="vertical-align: top;">Reduction by 1/2, Applicant qualifies for Small Entity Status.</td> <td style="text-align: center; vertical-align: bottom;">\$</td> </tr> <tr> <td colspan="3" style="text-align: right; font-weight: bold;">SUBTOTAL =</td> <td style="text-align: center;">\$ 990.00</td> </tr> <tr> <td colspan="3" style="vertical-align: top;">Processing fee of \$130 for furnishing the English translation later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))</td> <td style="text-align: center; vertical-align: bottom;">\$</td> </tr> <tr> <td colspan="3" style="text-align: right; font-weight: bold;">TOTAL NATIONAL FEE =</td> <td style="text-align: center;">\$ 990.00</td> </tr> <tr> <td colspan="3" style="vertical-align: top;">Fee of \$40.00 for recording the enclosed assignment (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).</td> <td style="text-align: center; vertical-align: bottom;">\$</td> </tr> <tr> <td colspan="3" style="text-align: right; font-weight: bold;">TOTAL FEES ENCLOSED =</td> <td style="text-align: center;">\$ 990.00</td> </tr> <tr> <td colspan="3"></td> <td style="text-align: right;">Amt. to be refunded: \$</td> </tr> <tr> <td colspan="3"></td> <td style="text-align: right;">Amt. charged: \$</td> </tr> </table>	Claims	Number Filed	Number Extra	Rate	Total Claims	13 - 20 =	-0-	x \$18.00	Independent Claims	2 - 3 =	-0-	x \$80.00	Multiple Dependent Claim(s) (if applicable)			+ \$270.00	TOTAL OF ABOVE CALCULATIONS =			\$ 990.00	Reduction by 1/2, Applicant qualifies for Small Entity Status.			\$	SUBTOTAL =			\$ 990.00	Processing fee of \$130 for furnishing the English translation later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))			\$	TOTAL NATIONAL FEE =			\$ 990.00	Fee of \$40.00 for recording the enclosed assignment (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).			\$	TOTAL FEES ENCLOSED =			\$ 990.00				Amt. to be refunded: \$				Amt. charged: \$		
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a. ☒ A check in the amount of \$ 990.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 06-1358 in the amount of \$ --- to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. 06-1358. A duplicate copy of this sheet is enclosed.

SEND ALL CORRESPONDENCE TO:

Jacobson, Price, Holman & Stern, PLLC

400 7th Street, N.W., Suite 600
Washington, DC 20004
202-638-6666

CUSTOMER NUMBER: 00136

By

William E. Player
Reg. No. 31,409

JPH&S 3/95

A Method for Stimulating the immune system

Two different approaches have been used in the prior art to enhance the immune response against neoplastic cells. One approach uses the addition of cytokines like interleukin-2 (IL-2) or transfection of tumor cells and/or immune cells with genes coding for cytokines like IL-2 or other proteins enhancing the immune response like transfection of tumor cells with lymphotactin or like transfection of T-lymphocytes with CD-40 Ligand.

The second approach uses the inhibition of immunosuppressive molecules to enhance the body's immune response to tumor cells. Thus, J. NEUROSURG. 78 (1993) 944-51, Jachimczak et al. (1993) and WO 94/25588, Schlingensiepen et al. (1994) teach the use of antisense oligonucleotides targeted to TGF- β to reverse tumor-induced immunosuppression.

Several documents in the prior art teach that a combination of these two approaches is either not efficacious or is not beneficial over use of one of the two approaches used alone.

Thus, CANCER BIOTHER. 8(2), 1993, 159 - 170, Gridley et al., as well as CANCER BIOTHER. 9(4), 1994, 317-327, Mao et al., both teach that a combination of anti-transforming growth factor-beta antibody with IL-2 does not cause significant antitumor effects.

Furthermore, PROC. NATL. ACAD. SCI 93, (1996), 2909-2914, Fakhrai et al., teaches that a combination of transfection with genes encoding antisense sequences to transforming growth factor beta (TGF- β) TGF- β mRNA with transfection of IL-2 into tumor cells does not increase the immune response against the tumor compared to transfection with TGF- β antisense alone.

Surprisingly, in contrast, certain combinations of stimulators and inhibitors are more efficacious than either approach alone.

The present invention discloses a medicament comprising a combination of

- at least one inhibitor of the effect of a substance negatively effecting an immune response, the substance selected from the group consisting of TGF- β and its receptors, VEGF and its receptors, interleukin 10 (IL-10) and its receptors, PGE₂ and its receptors, wherein the inhibitor has a molecular weight of less than 100 kDa and
- at least one stimulator positively effecting an immune response.

In a preferred embodiment, the inhibitor is inhibiting the synthesis or function of molecules suppressing or downregulating or negatively affecting the immune response. The inhibitor can be an oligonucleotide which may function as an antisense nucleotide or a ribozyme or it may be an antibody fragment derived from an antibody e.g. a fab-fragment or a single chain antibody.

Preferably, the stimulator is positively effecting the immune response by increasing presentation of antigens and/or enhancing proliferation and/or function of immune cells.

In a preferred embodiment, the stimulator is enhancing the synthesis or function of molecules stimulating, enhancing, upregulating and/or positively regulating the immune response. In particular, the stimulator is stimulating and/or enhancing the synthesis and/or the function of factors such as GM-CSF, SCF, CSF, IFN- γ , FLT-3-ligand as well as monocyte chemotatic proteins (MCP-1), interleukin-2, interleukin-4, interleukin-12 and/or interleukin-18 or is one of the mentioned interleukins or is selected from the group consisting of viruses, viral antigens, antigens expressed in tumor cells or pathogens but not in normal cells, organspecific antigenes expressed in affected organs which are not essential for the organism, e. g. prostate, ovary, breast, melanine producing cells.

The stimulators are preferably selected from

- a) Chemokines, including lymphotactin and/or immune cell attracting substances and/or
- b) viruses and/or parts of viruses, including retroviruses, adenoviruses, papillomaviruses, Epstein-Barr-Viruses, Viruses that are non-pathogenic including Newcastle-Disease virus, Cow-pox-virus and/or
- c) autologous and/or heterologous MHC-Molecules and/or
- d) molecules involved in antigen processing and/or
- e) molecules involved in antigen presentation and/or
- f) molecules involved in mediating immune cell effects and/or
- g) molecules involved in mediating immune cell cytotoxic effects and/or

- h) molecules involved in antigen transportation and/or
- i) co-stimulatory molecules
- j) peptides enhancing recognition by immune cells and/or cytotoxic effects of immune cells
- k) the peptides containing one or more amino acids differing between a protein in the target cell from the other cells within an organism
- l) the peptides according to j) being
- Peptides containing one or more mutations and/or amino acid substitutions of the ras protein amino and/or
 - Peptides containing one or more mutations and/or amino acid substitutions of the p53 protein and/or
 - Peptides containing one or more mutations and/or amino acid substitutions of the EGF-Receptor protein and/or
 - Peptides containing one or more mutations and/or amino acid substitutions of fusion peptides and/or fusion proteins and/or
 - Peptides containing one or more mutations and/or amino acid substitutions and/or amino acid substitutions caused by gene rearrangements and/or gene translocations and/or
 - Peptides containing one or more mutations and/or amino acid substitutions of the retinoblastoma protein and/or

- Peptides containing one or more mutations and/or amino acid substitutions of proteins coded by oncogenes and/or protooncogenes and/or
- Peptides containing one or more mutations and/or amino acid substitutions of proteins coded by anti-oncogenes and/or tumor suppressor genes and/or
- Peptides derived from proteins differing in the target cell by one or more amino acids from the proteins expressed by other cells in the same organism and/or
- Peptides derived from viral antigens and/or coded by viral nucleic acids and/or
- Peptides derived from proteins expressed in a diseased organ but not in the nervous system, muscle, hematopoietic system or other organs essential for survival. Diseased organs are e. g. prostate, ovary, breast, melanine producing cells and the like.

m) tumor cell extracts and/or tumor cell lysates and/or adjuvants,

n) fusion cells of dendritic and tumor cells.

These fusion cells are hybridoma cells derived from a mixture of dendritic cells and tumor cells. Dendritic cells are generated e. g. by treatment of PBMC with GM-CSF and IL-4 or a mixture of GM-CSF, IL-4 and IFN- γ or FLT-3 ligand. Fusion of dendritic cells with tumor cells can be achieved e. g. using PEG (polyethylene glycole) or electrofusion.

Surprisingly, treatment of PBMC with VEGF-oligonucleotides enhanced the number and/or effectiveness of dendritic cells.

In one embodiment of the invention the inhibitor is an oligonucleotide. Preferably the oligonucleotides of Fig. 1 are useful in the medicament of the present invention.

In a further embodiment, the invention provides oligonucleotides having one of the sequences given in figure 1-2 to 1-4.

Also oligonucleotides having 1 to 10 additional nucleotides at the 5'- or 3'- end are part of the invention.

Oligonucleotide sequences used for transfection are usually much longer sequences than those used for antisense oligonucleotides, which usually do not exceed 30 bases in length and are applied as short single-stranded sequences and are not integrated into a vector system.

Since transfected sequences are usually much longer than oligonucleotides, if cross inhibition of different members of a protein family would occur with the antisense technology, such cross inhibition of other mRNAs than the target mRNA, is much more likely with transfected antisense sequences, compared to oligonucleotides. However, Cell Growth Differ, Vol. 6(12), February 1995, pages 1635 - 1642, Huang, F. et al. teaches "only the K6 transfectant exhibited 39 and 33% respectively of the levels of TGF beta1 mRNA and active secreted TGF beta1 protein of the parental line. K6 exhibited no change in TGF beta2 expression and TGF beta3 expression was not detected in either parental or transfectant cell line."

It was therefore surprising to find oligonucleotides according to this invention, which were able to significantly reduce expression of both, TGF- β_1 as well as TGF- β_2 e. g. TGF- β_1 -14, TGF- β_1 -15, TGF- β -17-c-2260, TGF- β -123-2262, TGF- β -23-2268, TGF- β_2 -4, TGF- β_2 -14, TGF- β_2 -15, TGF- β_2 -9, TGF- β_2 -14/1, TGF- β_2 -14/2, TGF- β_1 -136. Furthermore surprisingly oligonucleotides were designed, which were

able to significantly reduce expression of TGF- β_2 as well as TGF- β_3 .

Surprisingly even oligonucleotides were found, which were able to significantly reduce expression of TGF- β_2 as well as TGF- β_1 and TGF- β_3 , e. g. b1-N17, b1-N14, b1-N24, TGF- β_2 -9, TGF- β_2 -14, TGF- β_2 -15, TGF- β -17-c-2260, TGF- β -12-9/20-2261, TGF- β -123-2262, TGF- β -12-9/22-2263, TGF- β -23-2268, TGF- β 1-98-11, TGF- β 1-98-23, TGF- β 3-98-7, TGF- β 3-98-10, TGF- β -1-rwk-5, TGF- β -3-rwk-2, TGF- β -1-rwk-5, TGF- β -3-rwk-9, TGF- β -3-rwk-23, TGF- β 1-3, TGF- β 1-10.

Thus oligonucleotides which are effective against expression of at least two of TGF- β_1 , TGF- β_2 and/or TGF- β_3 are also part of the invention.

These findings were also surprising in view of the fact that sequence comparison between the mRNAs of TGF- β_2 , TGF- β_1 and TGF- β_3 showed that not a single sequence of 20 bases in length could be found that would be identical within the three different mRNAs. Even if such a hypothetical sequence had really existed, inhibition of the three mRNAs by such a hypothetical consensus sequence would have been extremely unlikely, since it is well known in the art that only a small minority of antisense sequences complementary to a certain mRNA actually exert a so-called antisense effect, *i.e.* inhibit expression of the respective protein.

Endothelial synthesis of monocyte chemotactic protein-1 (MCP-1) has been implicated in the regulation of monocyte recruitment for extravascular pools both under physiological and inflammatory conditions.

MCP-1 antisense oligonucleotides were able to modulate monocyte infiltration and were thus anti-inflammatory.

These antisense-oligonucleotides are useful for the treatment of inflammatory diseases e.g. asthma, morbus crohn, collitis ulcerosa, diabetes, glomerulonephritis, acute respiratory distress syndrome and artherosclerotic plaque formation.

In a preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids have modifications at the bases, the sugars and/or the phosphate moieties of the oligonucleotides.

In a further preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids have modifications wherein the modifications are phosphorothioate (S-ODN) internucleotide linkages and/or methylphosphonate internucleotide linkages and/or phosphoramidate linkages and/or peptide linkages and/or 2'-O-derivatives, such as 2'-O-methyl or 2'-O-methoxyethoxy modifications of the sugar and/or modifications of the bases.

In a further preferred embodiment of the invention the oligonucleotides and/or ribozymes and/or nucleic acids are coupled to or mixed with folic acid, hormones, steroid hormones such as oestrogene, progesterone, corticosteroids, mineral corticoids, peptides, proteoglycans, glycolipids, phospholipids, polyethylene imine or other poly cations and derivatives therefrom.

Furthermore, the present invention provides a method of treating hyperproliferative diseases, neoplasms or infectious diseases by administering a medicament of the invention to patients in need thereof. The method is especially useful for the treatment of leukemia, non-hodgkin lymphoma, hodgkin lymphoma, bronchial carcinoma, esophageal carcinoma, colorectal carcinoma, gastric carcinomas, intestinal tumors, hepatic tumors, gall bladder and gallduct carcinomas, pancreatic carcinoma, anal carcinoma, breast cancer, ovarian carcinoma, cervial carcinoma, endometrium carcinoma, prostatic carcinoma, bladder carcinoma, malignant melanoma, brain tumors, and sarcomas.

The necessary doses of the medicament of the present invention depend on the disease and the severity of the disease. Whereas higher levels are more effective, they often have a higher degree of side effects. Suitable doses are selected to obtain concentrations of the oligonucleotides in the range of 0.1 to 10 $\mu\text{mol/l}$ and concentrations of the cytokines in the range of 10 to 1.000 U/ml in the patient blood.

In a preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ and the stimulator positively effecting an immune response is applied systemically (e.g. i.v. or s.c. or orally).

In another preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied systemically (e.g. i.v. or s.c. or orally) to the tumor and the stimulator positively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ.

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In another preferred embodiment of the invention the inhibitor of the effect of a substance negatively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ and the stimulator positively effecting an immune response is applied locally to a tumor or other pathologically affected site or organ.

Fig. 1 shows oligonucleotides useful in the present invention.

Fig. 2A shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 2 secretion in glioma cells in 10% MEM Dulbecco medium (3 day incubation with oligonucleotides).

Fig. 2B shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 3A shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 3B shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 2 secretion in glioma cells in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 4A shows TGF- β 1 concentration (ELISA) in glioma cells (3 day incubation with oligonucleotides).

Fig. 4B shows TGF- β 2 concentration (ELISA) in glioma cells (3 day incubation with oligonucleotides).

Fig. 5 shows lysis of tumor-cells: LAK-Cytotoxicity, Ratio of glioma-cells/PBMC: 1 : 20.

Fig 6A shows dendritic cells generated from PBMC (% of control). Cytokines: GM-CSF (400 U/ml) + IL-4 (300 U/ml).

Fig. 6B shows lysis of tumor-cells: Effects of 5 μ M VEGF-Antisense-Oligos on LAK-Cytotoxicity. Ration of tumor-cells/DC/PBMC was 1 : 5 :20.

Fig. 7A shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 1 secretion in PBMC in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 7B shows effects of oligonucleotides (f.c. 5 μ M) on TGF- β 2 secretion in tumor cells in 10% FCS RPMI 1640 medium (3 day incubation with oligonucleotides).

Fig. 8 shows lysis of tumor-cells: Effects of oligonucleotides on LAK-Cytotoxicity. Ration of tumor-cells/PBMC was 1 : 20.

Examples

Preparation of PBMC and tumor cells

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood of healthy donors by standard Ficoll-Hypaque gradient centrifugation. Briefly, heparinized blood was mixed with equal volumes of complete medium (CM: RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 1 mM L-Glutamine) and layered onto a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. After centrifugation at 400g for 30 min at room temperature, PBMCs banded at the plasma-Ficoll interface were recovered, washed three times and resuspended in complete medium. Cell viability, as determined by Trypan blue exclusion, was greater than 97%.

Human glioma cell lines were established from tumor specimens of patients with anaplastic astrocytoma (WHO Grad III) or from glioblastoma (WHO Grad IV).

Measurement of cell proliferation

For PBMC-proliferation assays (3H-thymidine incorporation and cell counting), freshly isolated PBMCs were cultured for 72h in 96-well round-bottom plates (Nunc, Copenhagen, Denmark) at a final concentration (f.c.) of 10^5 cells/well (100 μ L CM). For cell number determination the cells were counted by hemacytometer. Cell viability was determined by trypan blue staining. Treated and untreated cells showed 95-100% viability after 72h *in vitro* growth (with or without S-ODN).

For the tumor proliferation experiments $10^4/100$ μ L glioma cells were seeded into 96-well flat-bottom plates (Nunc, Denmark) and incubated with cytokines and/or oligonucleotides. The DNA synthesis rate was measured by a standard 3H-thymidine incorporation assay and determination of cell number was performed as described above.

Quantification of TGF- β 1 protein in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

The culture medium was harvested after 3 days, cleared of cellular components by centrifugation, filtered and stored at -70°C until processed further. TGF- β 1 and TGF- β 2 concentrations were measured after acidification of supernatants by TGF- β 1 and TGF- β 2 ELISA (R&D Systems, Minneapolis, USA) in duplicates, as recommended by the manufacturer.

Figures 1 - 4 and 7 show the effect of oligonucleotides on the TGF- β secretion in cells. The concentration of the TGF- β is reported as an optical density. The higher the optical density the higher is the concentration of the TGF- β .

Figure 1A and 1B shows the effect of the oligonucleotides on the TGF- β secretion. Control oligos (GAA GGA ATT ACC ACT TTC) have no effects whereas the oli-

gonucleotides shown in the figures reduce the secretion of TGF- β . The oligos in figure 1 are more effective against TGF- β 1.

Figure 2 shows further oligos and their effects on TGF- β secretion. TGF- β -14 is especially effective against the secretion of TGF- β 1 and - β 2.

Figure 3 shows further oligonucleotides being effective against secretion of TGF- β 1 and - β 2. These oligonucleotides are more effective against TGF- β 2 but are also effective against TGF- β 1.

Figure 8 shows a supra additive effect on tumor cell cytotoxicity by a combination of 2 μ M each of a TGF- β 1 and TGF- β 2 antisense oligonucleotide compared to a single 5 μ M dose of either oligonucleotide.

CARE-LASS (calcein-release-assay) to measure cytotoxic PBMC activity

A standard calcein-release-assay (CARE-LASS assay) to determine cytotoxic activity of PBMC was employed as described by Lichtenfels, R., Biddison, W.E., Schulz, H., Vogt, A.B. and R. Martin. CARE-LASS (calcein-release assay), an improved fluorescence-based test system to measure cytotoxic lymphocyte activity. J. Immunol. Meth., 172: 227-239, 1994.

Target and Effector cells

At the day of the assay malignant glioma were harvested, washed twice in 5% FCS /PBS and incubated with Calcein-AM (Molecular Probes, USA) for 30min in 37°C. Labeled target cells were washed twice in 5% FCS/PBS, adjusted to 100 000 / ml, and plated into 96-well U-shaped microtiter plates (Nunc, Denmark) at the final volumen of 100uL/well.

PBMC were washed with 5% FCS/PBS and adjusted to final concentration of 1-10 Mio cells /ml.

Cells were treated with cytokines and oligodeoxynucleotides as described in the individual experiments.

Assay

To measure CTL activity effector cells were plated into 96-well U-shape microtiter plates at Target : Effector Ratios of 1:10 - 1:100. To measure spontaneous release and total release of calcein, wells were preloaded with 200uL 5%FCS/PBS or 200uL lysis buffer (50mM sodium-borate, 0.1% Triton, pH 9.0) respectively. After incubating the plate for 4 h at 37°C in an incubator, 100uL of supernatans were transferred into new wells and measured employing an automated fluorescence scanner (Titertek Fluoroskan II, Germany). Both for excitation and for emission, filter settings 2 were chosen (ex 2 - 485nm, em 2 -538 nm). The percent of cytotoxicity was determined from the following equation:

F/CTL assay - F spontaneous release

----- x 100 = % cytotoxicity

F total lysis - F spontaneous release

In one set of experiments, glioma cells, denritic cells (DC) and PBMC were co-cultured. In these experiments DC were generated from PBMC using the cytokines GM-CSF and IL4. Cells were further treated with antisense VEGF-oligonucleotides according to the invention or with no oligonucleotides as control experiments. Tumor cells were also treated with the cytokines GM-CSF and IL4 with or without oligonudeotides.

PBMC were only treated with oligonucleotides according to the invention, but not with the cytokines GM-CSF and IL4. oligos were used at a concentration of 5 μ M unless indicated otherwise in the descriptions in the figures.

The CARE-LASS (calcein-release-assay) was used to measure cytotoxic PBMC activity.

In one set of experiments glioma cells and PBMC were treated either with a single oligonucleotide or with a combination of oligonucleotides. The single oligonucleotides were given at 5 μ M concentration. In the combination experiment, each oligonucleotide was given at 2 μ M concentration. Both, PBMC and tumor cells were incubated separately with the oligonucleotide(s) for 72 h.

The CARE-LASS (calcein-release-assay) was used to measure cytotoxic PBMC activity.

Claims

1. Medicament comprising a combination of
 - at least one inhibitor of the effect of a substance negatively effecting an immune response, the substance selected from the group consisting of TGF- β and its receptors, VEGF and its receptors, interleukin 10 (IL-10) and its receptors, PGE₂ and its receptors, wherein the inhibitor has a molecular weight of less than 100 kDa and
 - at least one stimulator positively effecting an immune response.
2. The medicament of claim 1 wherein the inhibitor is inhibiting the synthesis or function of molecules suppressing or downregulating or negatively affecting the immune response.
3. The medicament of claim 1 wherein the inhibitor is an oligonucleotide.
4. The medicament according to claim 3 wherein the oligonucleotide is an antisense nucleotide and/or a ribozyme.
5. The medicament according to claim 3 wherein the oligonucleotides has a sequence according to figure 1.
6. The medicament according to claim 1, wherein the inhibitor is a fab-fragment or single chain antibody (scFv).
7. The medicament according to claim 1, wherein the stimulator is enhancing the synthesis or function of molecules stimulating, enhancing, upregulating and/or positively regulating the immune response.

8. The medicament according to claim 7, wherein the stimulator is stimulating and/or enhancing the synthesis and/or the function of factors such as GM-CSF, SCF, CSF, IFN, FLT-3-ligand, monocyte chemotatic proteins (MCP-1), interleukin-2, interleukin-4, interleukin-12 and/or interleukin-18 or is one of the mentioned interleukins or is selected from the groups consisting of viruses, viral antigens, antigens expressed in tumor cells or pathogens, but not in normal cells, organ specific antigens expressed in affected organs which are not essential for the organism or fusion cell of dendritic and tumor cells.
9. The medicament according to claim 1, wherein the medicament comprises two or more of the inhibitors and/or the stimulators.
10. An oligonucleotide having one of the sequences of figures 1-2 to 1-4 (No. 55 - 213).
11. The oligonucleotide according to claim 10 wherein each oligonucleotide is effective against expression of at least two of TGF- β_1 , TGF- β_2 and/or TGF- β_3 having the sequence.
12. A method of treating neoplasm or infectious diseases by administering a medicament according to claim 1 to a patient in need thereof.
13. A method according to claim 12 for the treatment of hyperproliferative diseases, leukemia, non-hodgkin lymphoma, hodgkin lymphoma, bronchial carcinoma, esophageal carcinoma, colorectal carcinoma, gastric carcinomas, intestinal tumors, hepatic tumors, gall bladder and gallduct carcinomas, pancreatic carcinoma, anal carcinoma, mastocarcinoma, ovarian carcinoma, cervical carcinoma, endometrium carcinoma, prostatic carcinoma, bladder carcinoma, malignant melanoma, brain tumors, and/or sarcomas.

1. TGF-β2-1 C ACA CAG TAG TGC A
 2. TGF-β2-2 GC ACA CAG TAG TGC
 3. TGF-β2-3 GC TTG CTC AGG ATC TGC
 4. TGF-β2-4 TAC TCT TCG TCG CT
 5. TGF-β2-5 C TTG GCG TAG TAC T
 6. TGF-β2-6 G TAA ACC TCC TTG G
 7. TGF-β2-7 GT CTA TTT TGT AAA CCT CC
 8. TGF-β2-8 GC ATG TCT ATT TTG TAA ACC
 9. TGF-β2-9 CGG CAT GTC TAT TTT GTA
 10. TGF-β2-10 G GCA TCA AGG TAC C
 11. TGF-β2-11 CTG TAG AAA GTG GG
 12. TGF-β2-12 AC AAT TCT GAA GTA GGG T
 13. TGF-β2-13 T CAC CAA ATT GGA AGC AT
 14. TGF-β2-14 GCT TTC ACC AAA TTG GAA GC
 15. TGF-β2-15 CTG GCT TTT GGG TT
 16. TGF-β2-16 T CTG ATA TAG CTC AAT CC
 17. TGF-β2-17 T CCT AGT GGA CTT TAT AG
 18. TGF-β2-18 T TTT TCC TAG TGG ACT
 19. TGF-β2-19 C AAT TAT CCT GCA CAT TTC
 20. TGF-β2-20 GC AAT TAT CCT GCA CA
 21. TGF-β2-21 GC AGC AAT TAT CCT GC
 22. TGF-β2-22 TG GCA TTG TAC CCT
 23. TGF-β2-23 TG TGC TGA GTG TCT
 24. TGF-β2-24 CC TGC TGT GCT GAG TG
 25. TGF-β2-25 C TTG GGT GTT TTG C
 26. TGF-β2-26 T TTA GCT GCA TTT GCA AG
 27. TGF-β2-27 G CCA CTT TTC CAA G
 28. TGF-β2-14/1 CTT TCA CCA AAT TGG AAG
 29. TGF-β2-14/2 CAC CAA ATT GGA AGC
 30. TGF-β2-14/3 TCA CCA AAT TGG AAG C
 31. TGF-β2-15/1 CTC TGG CTT TTG GG
 32. TGF-β2-9/1 CGG CAT GTC TAT TTT G
 33. TGF-β1-1 CGA TAG TCT TGC AG
 34. TGF-β1-2 GTC GAT AGT CTT GC
 35. TGF-β1-3 CTT GGA CAG GAT CT
 36. TGF-β1-4 CCA GGA ATT GTT GC
 37. TGF-β1-5 CCT CAA TTT CCC CT
 38. TGF-β1-6 GAT GTC CAC TTG CA
 39. TGF-β1-7 CTC CAA ATG TAG GG
 40. TGF-β1-8 ACC TTG CTG TAC TG
 41. TGF-β1-9 GTA GTA CAC GAT GG
 42. TGF-β1-10 CAC GTA GTA CAC GA
 43. TGF-β1-11 CAT GTT GGA CAG CT
 44. TGF-β1-12 GCA CGA TCA TGT TG
 45. TGF-β1-13 TGT ACT CTG CTT GAA C
 46. TGF-β1-14 CTG ATG TGT TGA AGA ACA
 47. TGF-β1-15 CTC TGA TGT GTT GAA G
 48. TGF-β1-16 GGA AGT CAA TGT ACA G
 49. TGF-β1-17 CAT GTC GAT AGT CTT GCA
 50. TGF-β1-18 AGC TGA AGC AAT AGT TGG
 51. TGF-β1-19 GTC ATA GAT TTC GTT GTG
 52. TGF-β1-20 CTC CAC TTT TAA CTT GAG
 53. TGF-β1-21 TGC TGT ATT TCT GGT ACA
 54. TGF-β1-137 CGA TAG TCT TGC AG

55. b1-N17 TCC TCT TCG ACT GCT CTC
 56. b2-N14 CGA AGG TTA AAC CAC TTT CG
 57. b2-N24 GTG AGT CGT GTC GTC C

58. TGF-β2-98-1 CATCGTTGTCGTCG
 59. TGF-β2-98-2 CGCTTCTTCCGCCG
 60. TGF-β2-98-3 CGAAGGAGAGCCATTCG
 61. TGF-β2-98-4 CGATGTAGCG
 62. TGF-β2-98-5 CGTCAAATCG
 63. TGF-β2-98-6 CGTAGTACTCTTCGTCG
 64. TGF-β2-98-7 CGCGCTCGCAGGCG
 65. TGF-β2-98-8 CGGCCGCCCTCCGGCTCG
 66. TGF-β2-98-9 CGCGGATCGCCTCG
 67. TGF-β2-98-10 GAGCGCGACCGTGAC

68. TGF-β-17-c-2260 ACC TCC TTG GCG TAG TA
 69. TGF-β-12-9/20-2261 AGG GCG GCA TGT CTA TTT TG
 70. TGF-β-123-2262 CAG AAG TTG GCA TTG TAC
 71. TGF-β-12-9/22-2263 AGG GCG GCA TGT CTA TTT TGT A
 72. TGF-β-23-2268 TGG GAC ACG CAG CAA GG

73. TGF-β1-98-1 CGGGGGCGGGGCGGGG
 74. TGF-β1-98-2 CGGGGCGGGGCGGGGCG
 75. TGF-β1-98-3 CGGCGCCGCCGAGGCGCCCG
 76. TGF-β1-98-4 CCGAGGTCCTTGCGG
 77. TGF-β1-98-5 CGGCGGTGCCGGGA
 78. TGF-β1-98-6 CTCGGCGGCCGGTAG
 79. TGF-β1-98-7 CGCTAAGGCG
 80. TGF-β1-98-8 CCGCACAACTCCGG
 81. TGF-β1-98-9 GCGAGTCGCTGG
 82. TGF-β1-98-10 CGGTTGCTGAGGTATCG
 83. TGF-β1-98-11 CCGGGAGAGCAACACGG
 84. TGF-β1-98-12 CGCTTCTCG
 85. TGF-β1-98-13 CCATTAGCACGCGGG
 86. TGF-β1-98-14 CGGGCTCCG
 87. TGF-β1-98-15 CCGGCCACCCGGTCGCGG
 88. TGF-β1-98-16 CGAGCACGGCCTCG
 89. TGF-β1-98-17 CGGGCAGCGGGCCGGGCG
 90. TGF-β1-98-18 CGCGGATGGCCTCG
 91. TGF-β1-98-19 CGATGCGCTTCCG
 92. TGF-β1-98-20 CCCGCGGCCGGCGGG
 93. TGF-β1-98-21 CGCAGCCCGGAGGGCG
 94. TGF-β1-98-22 CGGCGCCCCCG
 95. TGF-β1-98-23 CGGCACTGCCGAGAGCGCG
 96. TGF-β1-98-24 CGGGGATGAAGGCGGCG
 97. TGF-β1-98-25 CGGGTCGGCGACTCCCG
 98. TGF-β1-98-26 CGCCTGAGGGACGCCG
 99. TGF-β1-98-27 AAGCGTCCCCGGCG
 100. TGF-β1-98-28 CGCGGGGCAGCGTCGCG
 101. TGF-β1-98-29 CCCC CGCCTCCGG
 102. TGF-β1-98-30 CGGCGGCGGCTCG
 103. TGF-β1-98-31 CGCTCCGGGCGGAGGCCG
 104. TGF-β1-98-32 CGGCCCCGCGGGCG
 105. TGF-β1-98-33 CGGACGGGGCGTCC
 106. TGF-β1-98-34 CGGCCGGGGCCCTCG

107.	TGF-β3-98-1	TCGAGCTTCCCCGA
108.	TGF-β3-98-2	CCCGGAGCCGAAGG
109.	TGF-β3-98-3	CCCGAGGAGCGGG
110.	TGF-β3-98-4	ACGCAGCAAGGCGA
111.	TGF-β3-98-5	CGGGTTGTCGAGCCG
112.	TGF-β3-98-6	CGGCAGTGCCCCG
113.	TGF-β3-98-7	CGGAATTCTGCTCG
114.	TGF-β3-98-8	TTCGTTGTGCTCCG
115.	TGF-β3-98-9	ATTCCGACTCGGTG
116.	TGF-β3-98-10	ACGTGGGTCATCACCGT
117.	TGF-β3-98-11	CGAAGAAGCG
118.	TGF-β3-312	CCT AAT GGC TTC CA
119.	VEGF-98-1	CGGCCGCGGTGTGT
120.	VEGF-98-2	CGGGAATGCTTCCGCCG
121.	VEGF-98-3	CGGCTCACCGCTCGGC
122.	VEGF-98-4	CACGTCTGCGGATC
123.	VEGF-98-5	CCCCGCATCGCATCAGGG
124.	VEGF-98-6	CGCCTTGCAACGCG
125.	VEGF-98-7	CCGACCGGGGCCGG
126.	VEGF-49	GTTTCATGGTTTCGG
127.	VEGF-55	GCAGAAAGTTCATGG
128.	VEGF-188	GCTGATAGACATCC
129.	VEGF-190	GCGCTGATAGACAT
130.	VEGF-194	GTAGCTGCGCTGATAG
131.	VEGF-253	CTCGATCTCATCAG
132.	VEGF-255	ATGTACTCGATCTCATC
133.	VEGF-260	GAAGATGTACTCGATC
134.	VEGF-263	CTTGAAGATGTACTCG
135.	VEGF-292	GCATCGCATCAGGG
136.	VEGF-294	CCGCATCGCATCAG
137.	VEGF-422	CATTTGTTGTGCTGTAGG
138.	VEGF-434	GGTCTGCATTCACATTTG
139.	VEGF-441	CTTTGGTCTGCATTG
140.	VEGF-445	CTTTCTTTGGTCTGC
141.	VEGF-450	GCTCTATCTTTCTTTGG
142.	VEGF-455	GTCTTGCTCTATCTTTC
143.	VEGF-459	CTTGTCTTGCTCTATC
144.	VEGF-596	CATCTGCAAGTACGTTTCG
145.	VEGF-598	CACATCTGCAAGTACGTT
146.	VEGF-600	GTCACATCTGCAAGTACG
147.	VEGF-600-2	CATCTGCAAGTACG
148.	VEGF-601	CACATCTGCAAGTAC
149.	VEGF-604	GTCACATCTGCAAG
150.	VEGF-607	CTTGTCACATCTGC
151.	VEGF-607-2	GGCTTGTCACATCTGC
152.	VEGF-610	CTCGGCTTGTCACATC
153.	VEGF-638	CTCCTTCCTCCTGC
154.	VEGF-766	GCT TGA AGA TGT ACCT CG
155.	VEGF-r-1062	CGT TGC TCT CCG ACG
156.	flt-1165	GAC ACG GCC TTT TCG
157.	flt-rm-2115	CCA GCA GCT GAC CAT GG
158.	flkl/kdr-m-2315	GAA ATC GAC CCT CGG
159.	MCP-1-Rec-A/B-571	GCA TGT TGT GGA TG
160.	MCP-1-1954	GCA GAG ACT TTC ATG C
161.	MCP-1-1955	ATA ACA GCA GGT GAC TGG

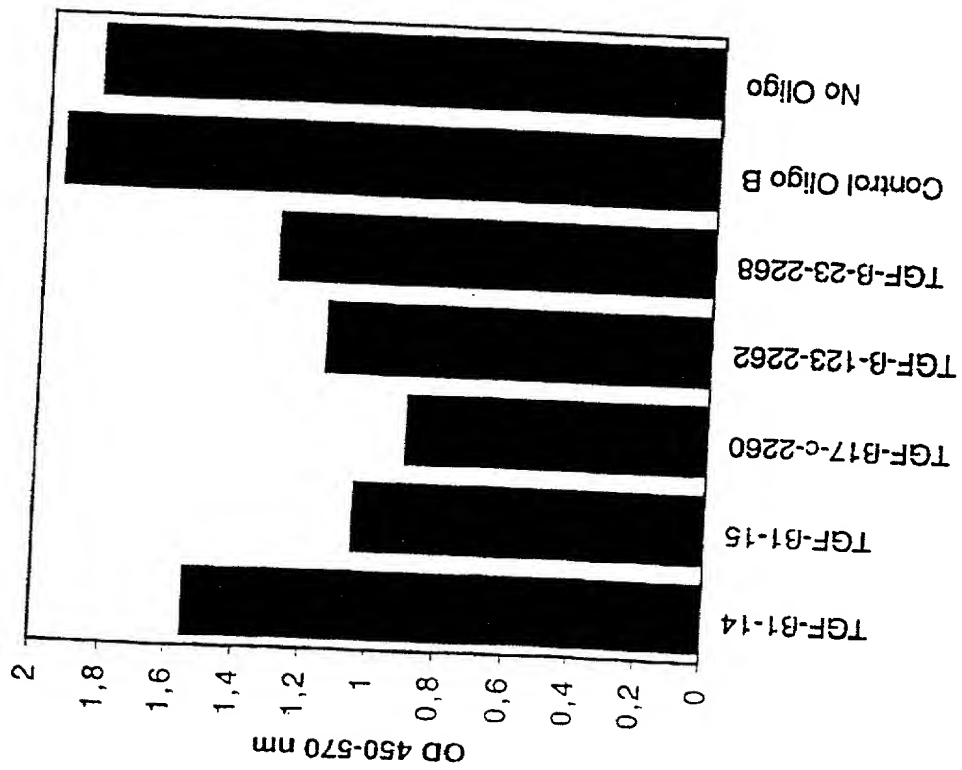
Fig. 1-3

162. MCP-1-1956
 163. MCP-1-2761
 164. MCP-1-2762
 165. VEGF-703
 166. flt-1567
 167. TGF- β -Rec-I-796
 168. TGF- β -1-rwk-1
 169. TGF- β -1-rwk-2
 170. TGF- β -1-rwk-3
 171. TGF- β -1-rwk-4
 172. TGF- β -1-rwk-5
 173. TGF- β -1-rwk-6
 174. TGF- β -1-rwk-7
 175. TGF- β -1-rwk-8
 176. TGF- β -1-rwk-9
 177. TGF- β -1-rwk-10
 178. TGF- β -1-rwk-11
 179. TGF- β -1-rwk-12
 180. TGF- β -1-rwk-13
 181. TGF- β -1-rwk-14
 182. TGF- β -1-rwk-15
 183. TGF- β -1-rwk-16
 184. TGF- β -1-rwk-17
 185. TGF- β -1-rwk-18
 186. TGF- β -1-rwk-19
 187. TGF- β -3-rwk-1
 188. TGF- β -3-rwk-2
 189. TGF- β -3-rwk-3
 190. TGF- β -3-rwk-4
 191. TGF- β -3-rwk-5
 192. TGF- β -3-rwk-6
 193. TGF- β -3-rwk-7
 194. TGF- β -3-rwk-8
 195. TGF- β -3-rwk-9
 196. TGF- β -3-rwk-10
 197. TGF- β -3-rwk-11
 198. TGF- β -3-rwk-12
 199. TGF- β -3-rwk-13
 200. TGF- β -3-rwk-14
 201. TGF- β -3-rwk-15
 202. TGF- β -3-rwk-16
 203. TGF- β -3-rwk-17
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 206. TGF- β -3-rwk-20
 207. TGF- β -3-rwk-21
 208. TGF- β -3-rwk-22
 209. TGF- β -3-rwk-23
 210. TGF- β -3-rwk-24
 211. TGF- β -3-rwk-25
 212. TGF- β -3-rwk-26
 213. TGF- β -3-rwk-27

GAA CCC ACT TCT GC
 GAC ACT TGC TGC TG
 CCA CTT CTG CTT GGG
 CTG CAA GTA CGT TCG
 TCC CTT ATG ATG CCA GCA AGT G
 CCA GCA ATG ACA GC
 G GGA AAG CTG AGG C
 T CGA GGG AAA GCT GA
 C CTC GAG GGA AAG C
 GG GCT GGT GTG GTG
 GA ACA GGG CTG GTG TG
 G AAC AGG GCT GGT G
 AG AGC GCG AAC AGG
 GA GAG CGC GAA CAG G
 CGA GAG CGC GAA CAG
 CCC CTG GCT CGG GGG
 C CCT GGC TCG GGG
 C CCC TGG CTC GGG G
 TCC CCC TGG CTC GG
 C TCC CCC TGG CTC G
 TGC GCT TCC GCT TCA C
 CC TCG ATG CGC TTC
 G ATG GCC TCG ATG C
 G GAT GGC CTC GAT GC
 ATG GCC TCG ATG CGC TT
 TC AGC AGG GCC AGG
 GCA AAG TTC AGC AGG GC
 GG CAA AGT TCA GCA GG
 GT GGC AAA GTT CAG CAG G
 GTG GCA AAG TTC AG
 GAC CGT GGC AAA GTT CAG
 AGA GAG GCT GAC CGT
 GAC AGA GAG AGG CTG AC
 A CAG AGA GAG GCT GA
 GT GGA CAG AGA GAG G
 CA AGT GGA CAG AGA GAG G
 TCT TCT TGA TGT GGC C
 CC CTC TTC TTC TTG ATG
 C ACC CTC TTC TTC T
 A TGG ATT TCT TTG GCA T
 GGA TTT CTT TGG C
 AA GTT GGA CTC TCT TCT C
 TAA GTT GGA CTC TCT TCT
 GAC CTA AGT TGG ACT C
 T TTC TAG ACC TAA GTT GG
 CT GAT TTC TAG ACC TAA G
 G AAG CAG TAA TTG GTG T
 GG AAT CAT CAT GAG G
 GGG AAT CAT CAT GAG
 G GTT GTC GAG CCG GT
 GTC CTC CCA ACA TAG TA
 GG GTC CTC CCA ACA

[illegible]

A.



6

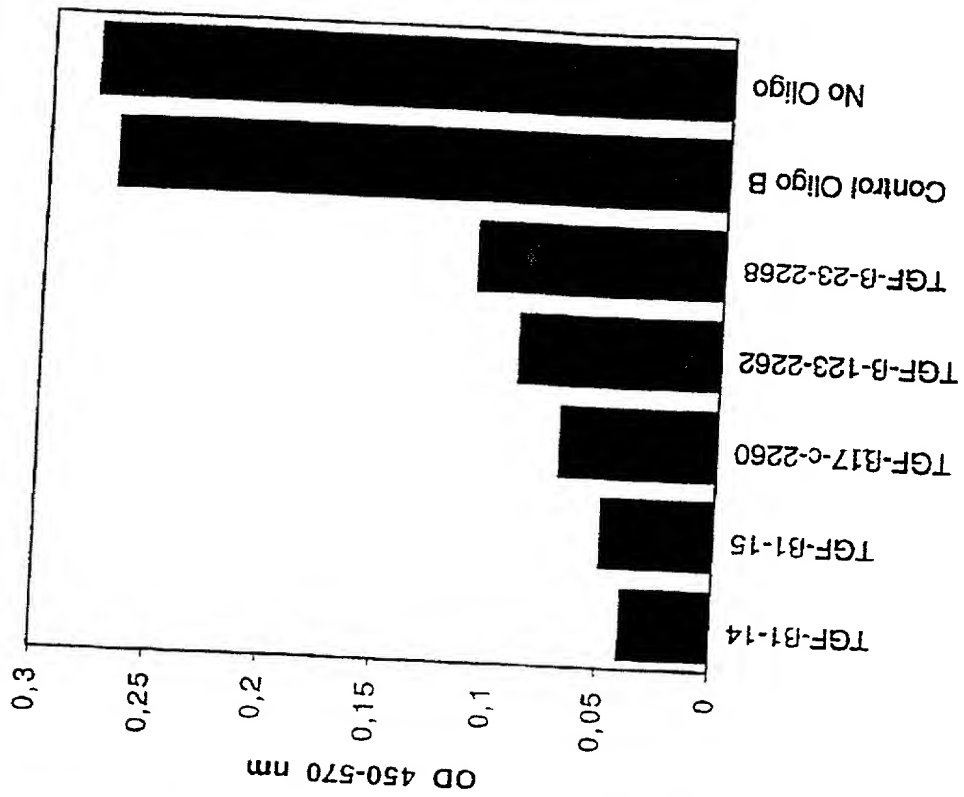


Figure 2

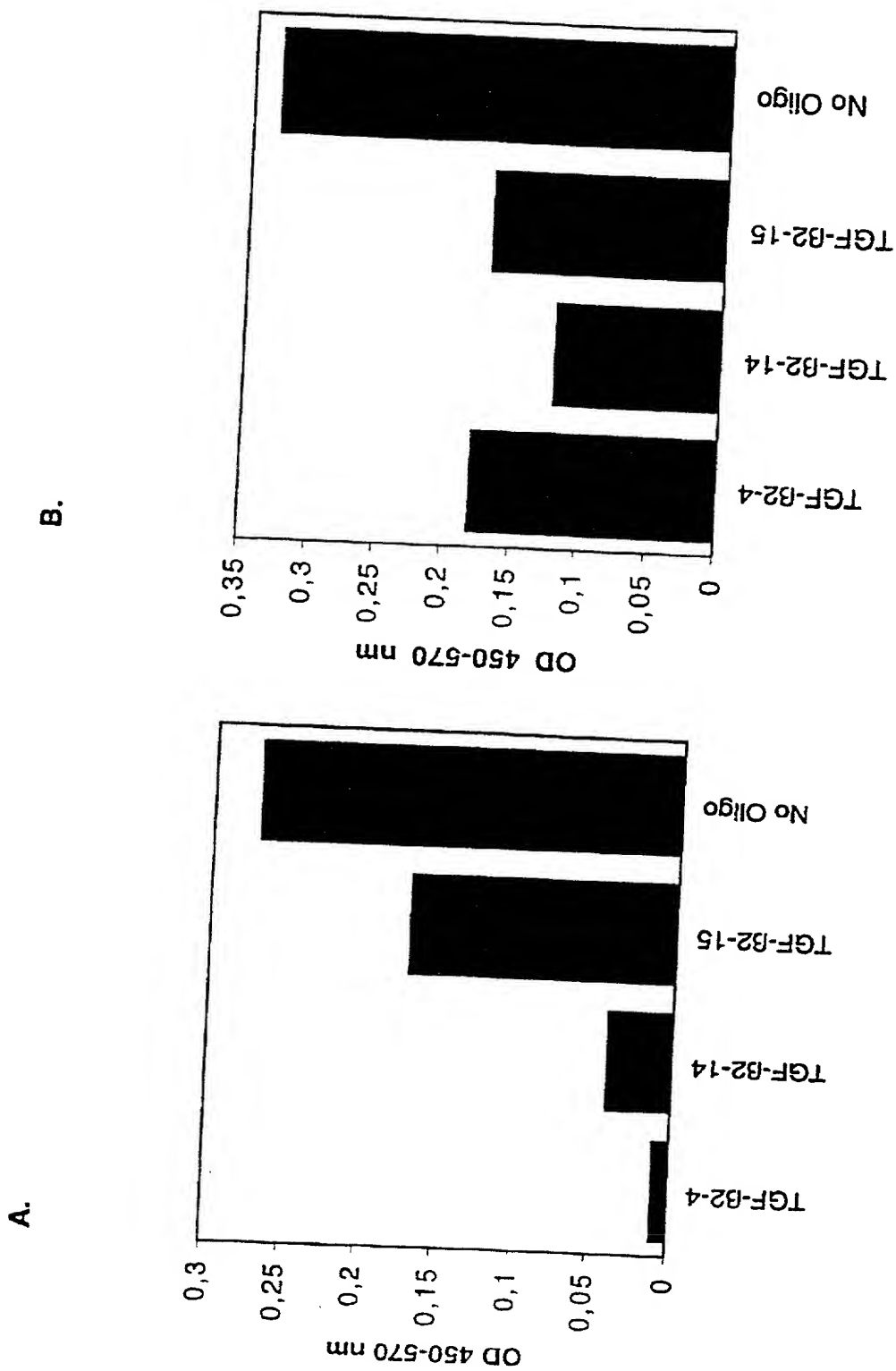
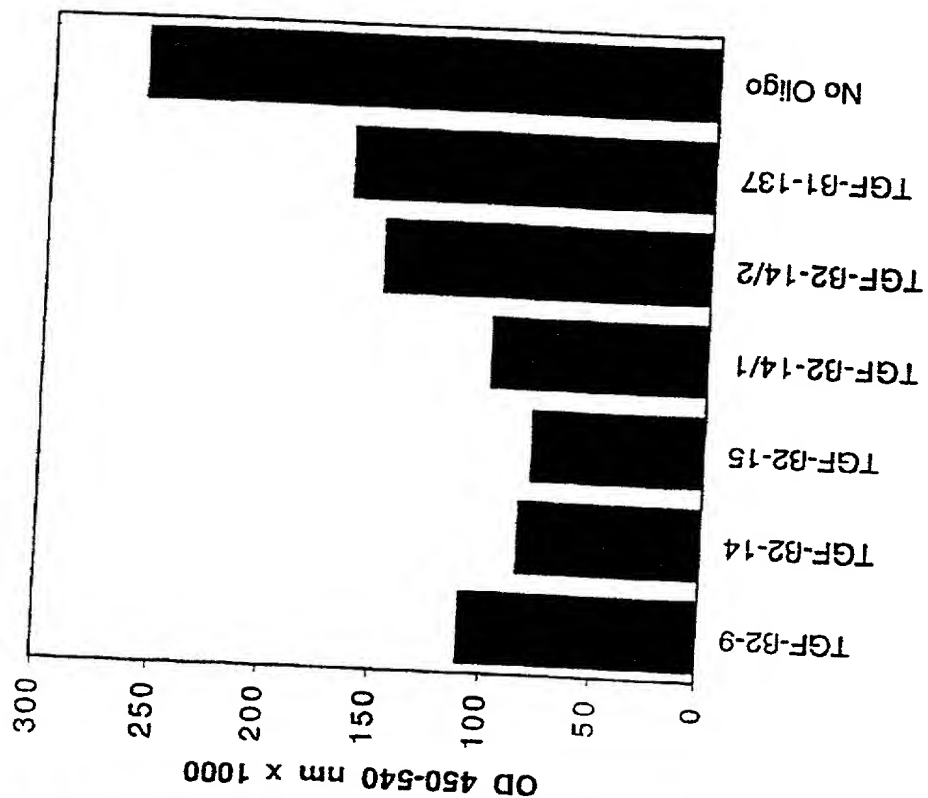


Figure 3

B.



A.

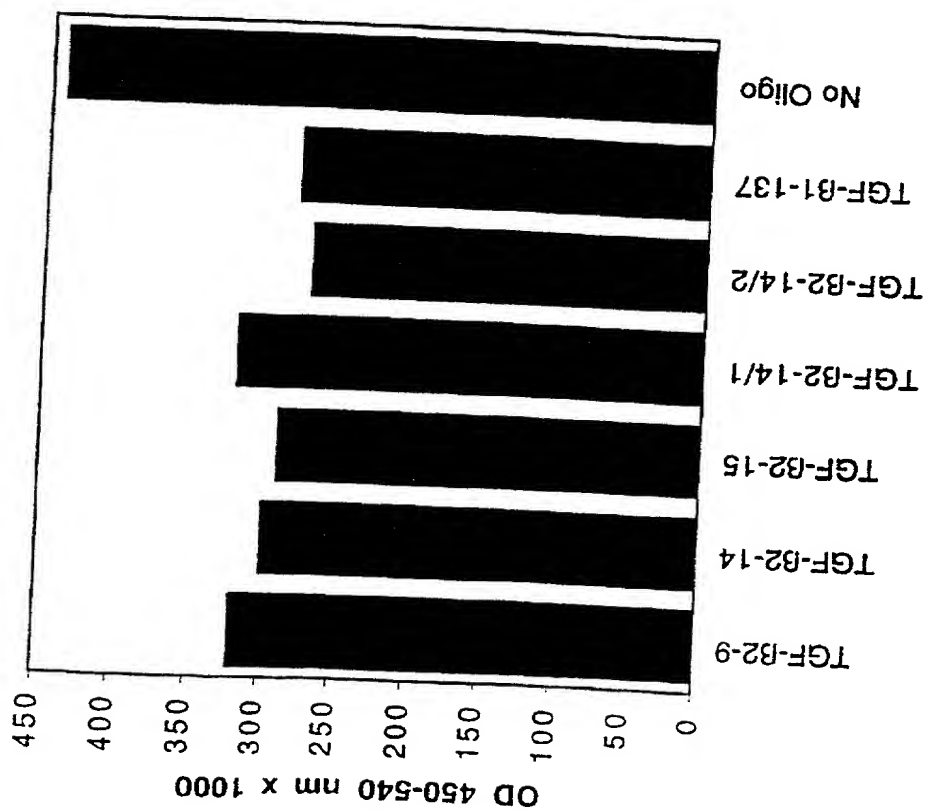


Figure 4

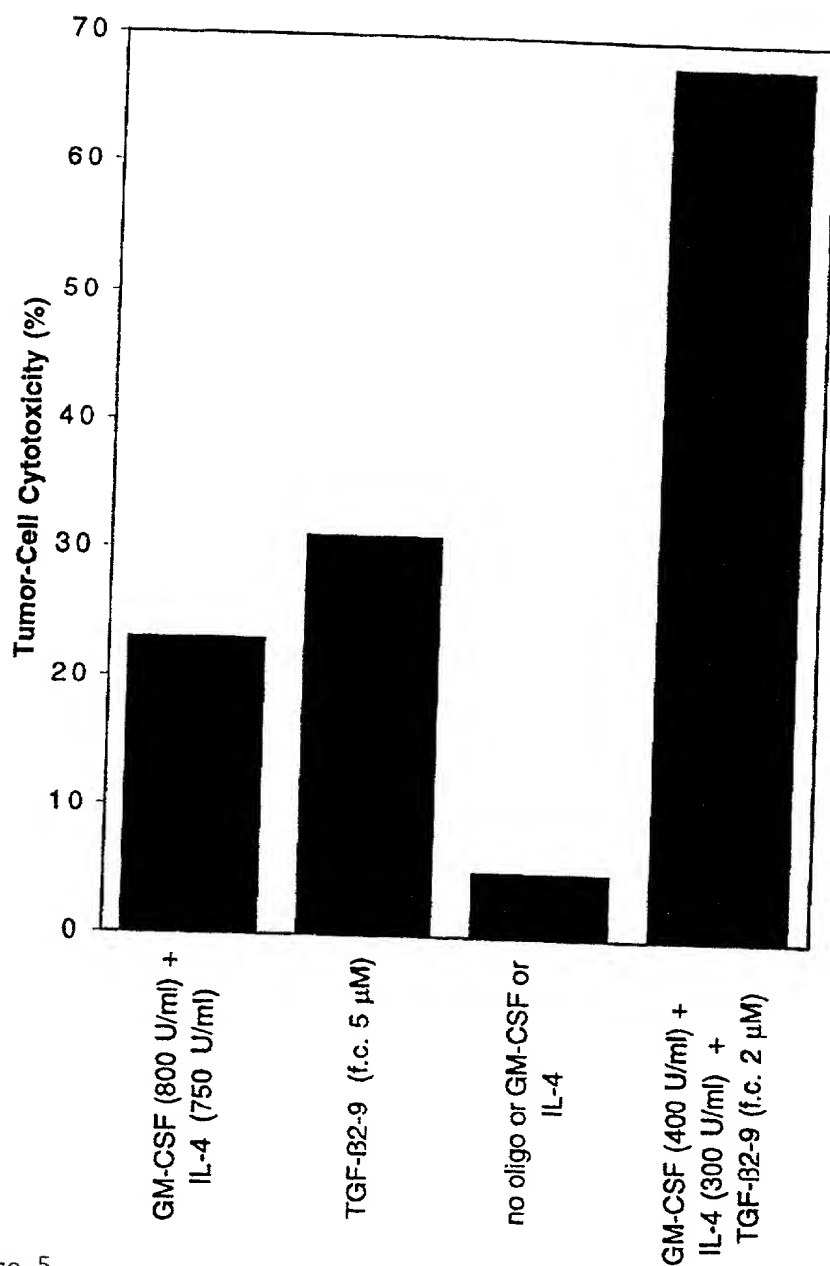
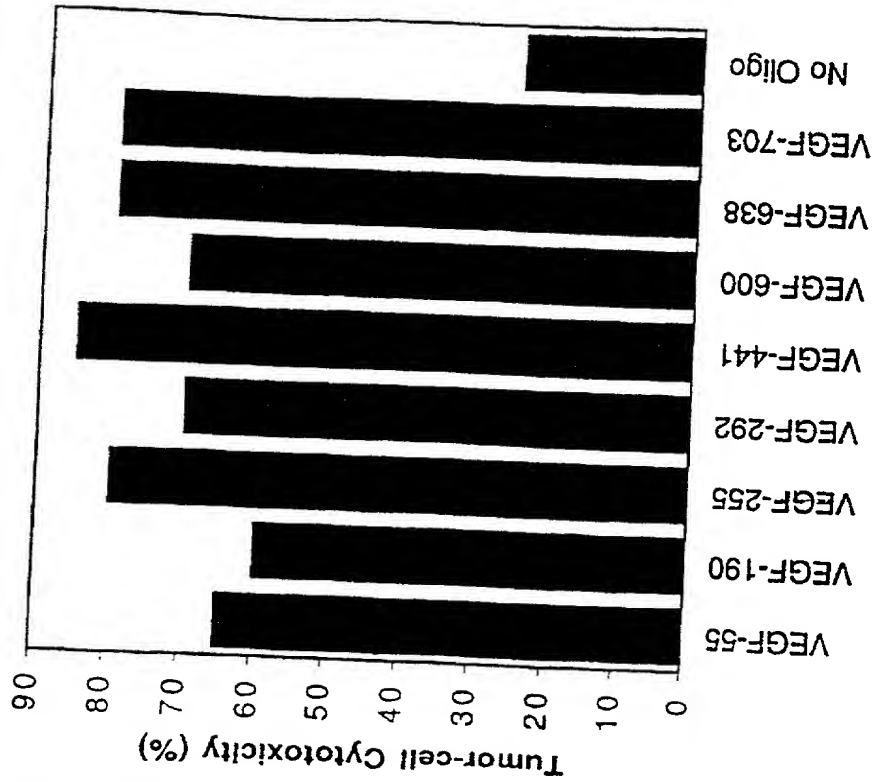


Figure 5

B.



A.

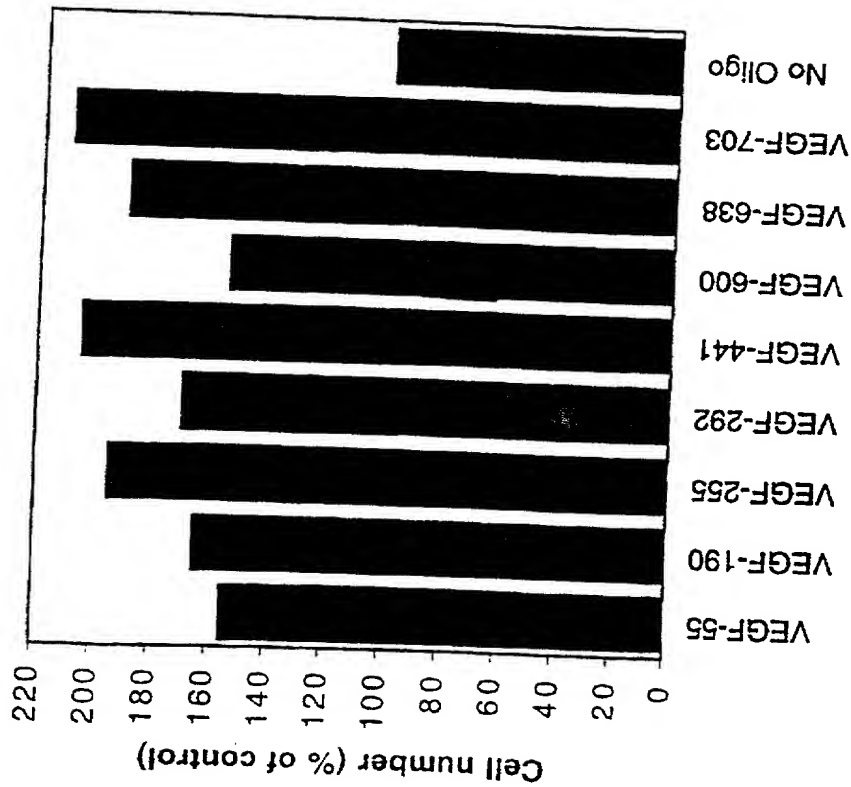


Figure 6

FIGURE 7A

A.

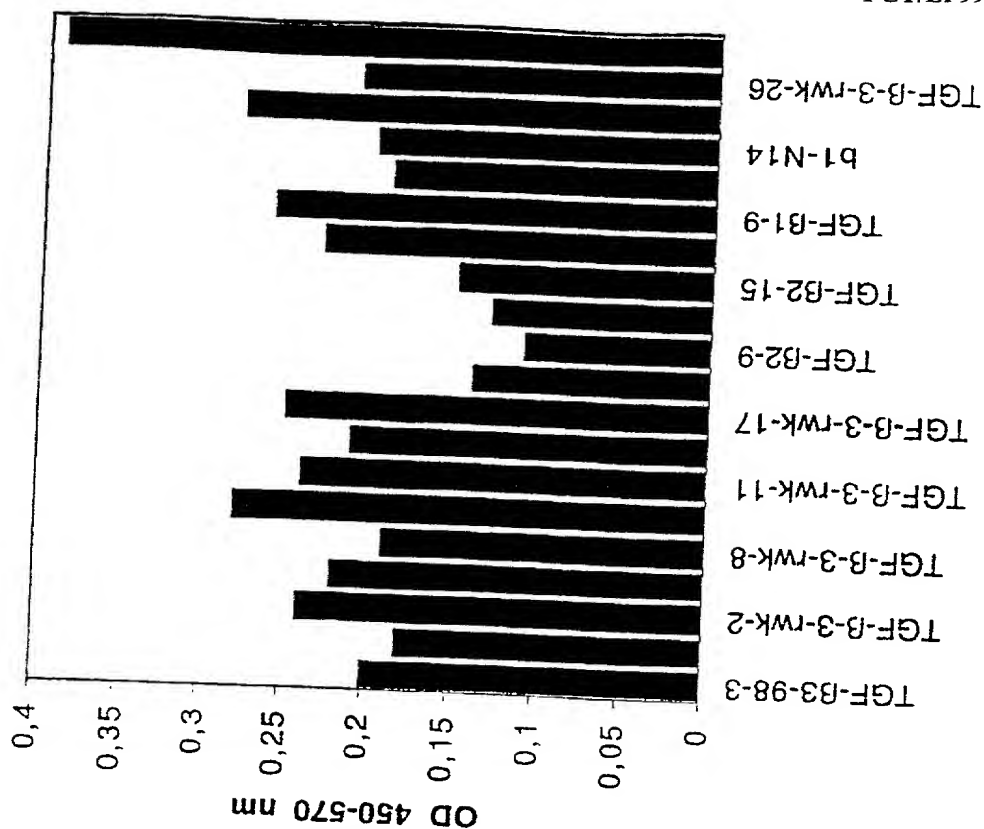
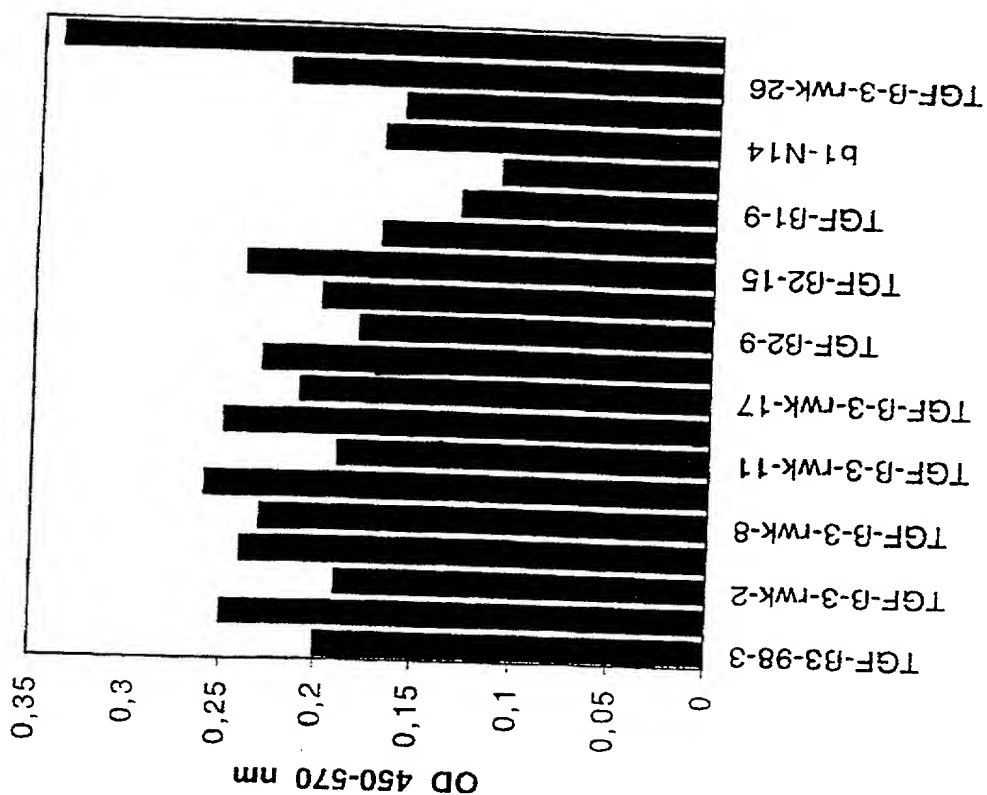


Figure 7

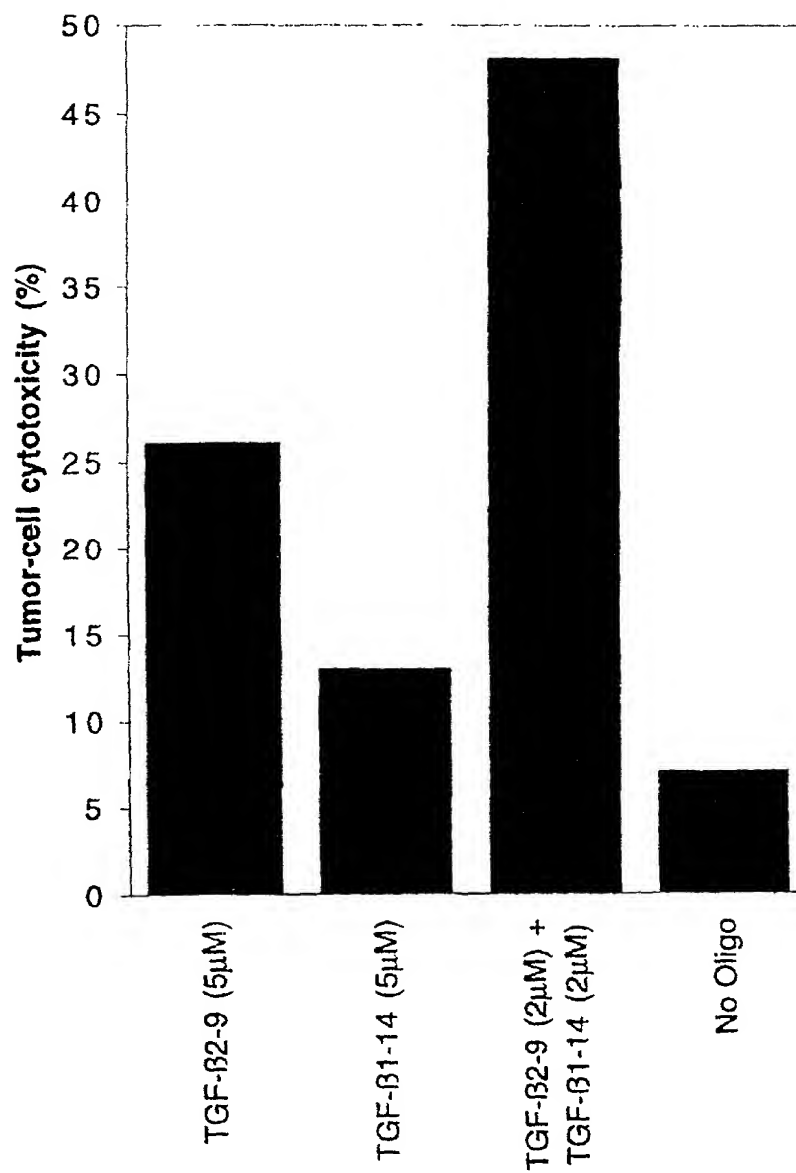


Figure 8